PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 72 (2000) 239–247

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Progestin regulation of 11β-hydroxysteroid dehydrogenase expression in T-47D human breast cancer cells

Felice Arcuri[a,*], Silvia Sestini[b], Claudia Ricci[a], Ylenia Runci[a], Antonietta Carducci[a], Luana Paulesu[c], Marcella Cintorino[a]

[a] *Institute of Pathological Anatomy and Histology, University of Siena, Siena, Italy*
[b] *Department of Molecular Biology, University of Siena, Siena, Italy*
[c] *Institute of General Physiology, University of Siena, Siena, Italy*

Received 7 October 1999; accepted 3 December 1999

Abstract

This study examined the enzymatic characteristics and steroid regulation of the glucocorticoid-metabolizing enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) in the human breast cancer cell line T-47D. In cell homogenates, exogenous NAD significantly increased the conversion of corticosterone to 11-dehydrocorticosterone, while NADP was ineffective. There was no conversion of 11-dehydrocorticosterone to corticosterone either with NADH or NADPH demonstrating the lack of reductase activity. In keeping with these results, RT-PCR analysis indicated a mRNA for 11β-HSD2 in T-47D cells, while 11β-HSD1 mRNA levels were undetectable. In T-47D cells treated for 24 h with medroxyprogesterone acetate (MPA), 11β-HSD catalytic activity was elevated 11-fold, while estrone (E_1), estradiol (E_2) and the synthetic glucocorticoid dexamethasone (DEX) were ineffective. The antiprogestin mifepristone (RU486) acted as a pure antagonist of the progestin-enhanced 11β-HSD activity, but did not exert any agonistic effects of its own. In addition, RT-PCR analysis demonstrated that MPA was a potent inducer of 11β-HSD2 gene expression, increasing the steady-state levels of 11β-HSD2 mRNA. Taken together, these results demonstrate that 11β-HSD2 is the 11β-HSD isoform expressed by T-47D cells under steady-state conditions and suggest the existence of a previously undocumented mechanism of action of progestins in breast cancer cells.

1. Introduction

In the mammary gland, proliferation, morphological and functional differentiation are controlled by steroid hormones. These endocrine effects are exerted through intracellular receptors that, after binding to the specific steroid, interact with regulatory sequences altering gene transcription rate [1]. Adrenal steroids control a variety of functions in normal and cancer breast epithelial cells. Glucocorticoids influence differentiation of mammary epithelial cells in primary culture [2] and are required for the initiation and maintenance of lactation [3,4]. The responsiveness of human breast cancer cells to steroids has been extensively investigated in vitro. Thus, glucocorticoid-dependent inhibition of cell growth has been demonstrated in breast cancer cell lines [5–7]. Other studies have shown that glucocorticoids modulate aromatase activity and can affect the rate of estrogen synthesis in breast carcinoma cells [8].

In glucocorticoid target tissues, intracellular hormone levels are modulated by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD), which catalyses the interconversion of 11-hydroxycorticosteroids, cortisol and corticosterone, to their 11-keto

* Corresponding author. Tel.: +39-0577-233-450; fax: +39-0577-233-235.
E-mail address: arcuri@unisi.it (F. Arcuri).

0960-0760/00/$ - see front matter
PII: S0960-0760(00)00039-X

metabolites, cortisone and 11-dehydrocorticosterone. To date, two distinct isoforms of 11β-HSD have been identified and cloned in mammals. The type 1 isoform (11β-HSD1) is an NADP-dependent enzyme found in most tissues, characterized by K_m values for its physiological substrates in the micromolar range and by acting as an oxoreductase [9]. In contrast, the type 2 isoform (11β-HSD2) is an NAD-dependent enzyme present in a limited range of tissues, showing a high affinity for its substrates and behaving as an exclusive dehydrogenase [10].

Several studies have evaluated the expression of 11β-HSD in the breast and its role as regulator of glucocorticoid action in epithelial and stromal cells. The presence of 11β-HSD activity was first reported in pregnant and lactating rat mammary gland where the enzyme, by reducing the intracellular levels of active glucocorticoids, is though to prevent premature milk production [11]. Immunohistochemical studies have indicated abundant 11β-HSD2 protein, colocalizing with the mineralocorticoid receptor, in normal and malignant human breast tissue [12]. Furthermore, the role of the enzyme as a modulator of the biological effects of glucocorticoids has been explored in human breast cancer and adipose stromal cells [13,14]. Despite the growing interest in the biological roles of mammary gland 11β-HSD, few data exist on factors regulating its expression. Importantly, a marked variation of 11β-HSD2 immunoreactivity among human breast tissues has been reported by Sasano et al. [12]. These authors hypothesized that alterations in the expression levels of breast epithelial cell 11β-HSD2 may occur in response to stimulatory factors [12]. Ovarian steroids are known to regulate 11β-HSD expression in several experimental models. We previously showed that in human endometrial stromal cells in culture, the levels of 11β-HSD increase after stimulation with medroxyprogesterone acetate (MPA) and that this effect is augmented by estradiol (E_2) [15]. Furthermore, it has been demonstrated that in human and murine endometrium, 11β-HSD activity varies during the menstrual cycle according to the secretion levels of estradiol and progesterone [16,17]. The present study was designed to evaluate the effect of ovarian steroids on the expression of 11β-HSD in breast cancer cells. To this aim, we developed a tissue culture system using the steroid receptor-positive, human breast cancer cell line T-47D. After examining the characteristics of 11β-HSD expressed by these cells, we studied the effects of estrogens and progestins on enzyme activity and mRNA levels. Our results demonstrate the presence of the 11β-HSD2 isoform in the T-47D cell line and provide direct evidence of increased enzyme expression following progestin treatment of the cells.

2. Materials and methods

2.1. Materials

NAD, NADP, NADH, NADPH, Triton X-100 and TLC plates (Polygram Sil G/UV;0.25 mm) were obtained from Sigma (St. Louis, MO, USA). Cell culture supplies were from Corning Costar (Acton, MA, USA).

2.1.1. Steroids

[1,2,6,7-^{3}H]Corticosterone (SA, 80 Ci/mmol) was purchased from Amersham International (Little Chalfont, UK). Non-radioactive steroids were obtained from Sigma Chemical. Mifepristone (RU486) was provided by Research Biochemicals International (Natick, MA, USA).

2.1.2. Preparation of [1,2,6,7-^{3}H]11-dehydrocorticosterone

The radioactive 11-dehydrocorticosterone was prepared following a procedure in use in our laboratory using a human prostate cancer cell line as a source of dehydrogenase activity [18]. Briefly, confluent cultures of LNCaP cells (American Type Culture Collection, Rockville, MD, USA) were harvested with a cell scraper and collected in ice-cold phosphate buffer solution (PBS). The cells were pelleted by centrifugation and suspended in 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.5), 0.2% (v/v) of Triton X-100 and sonicated. One mg of cell lysate was incubated overnight at 37°C with [1,2,6,7-^{3}H]corticosterone (50 μCi) and NAD (5 mmol/l) in 0.1 M sodium phosphate buffer (pH 7.5) in a final volume of 1.0 ml. The radioactive steroids were extracted with ethyl acetate and the product separated by TLC as described below. The band corresponding to 11-dehydrocorticosterone was scraped off of TLC plates and eluted with methanol. The steroid was collected by evaporating the solvent and suspended with methanol.

2.2. Methods

2.2.1. Cell cultures

The T-47D human breast carcinoma cell line was obtained from the American Type Culture Collection. Cells were grown at 37°C, in a 95% air: 5% CO_2 humidified incubator, in RPMI-1640. The medium was supplemented with 10% fetal bovine serum (FBS), L-glutamine 2 mmol/l, penicillin 100 U/ml, streptomycin 0.1 mg/ml, insulin 10 μg/ml, sodium pyruvate 1 mmol/l, HEPES 10 mmol/l, and sodium bicarbonate 0.2% (p/v) (Sigma).

2.2.2. Sample preparation and measurement of enzymatic activity

Cultures were rinsed three times with PBS, harvested by scraping with a rubber spatula and centrifuged at $1000 \times g$ for 5 min at 4°C. Cell pellets were suspended in ice-cold sodium phosphate buffer 0.1 M (pH 7.5), 0.2% (v/v) of Triton X-100, sonicated for 15 s on ice and divided in aliquots for total protein content and measurement of 11β-HSD activity. The latter was determined by measuring the conversion of corticosterone to 11-dehydrocorticosterone in the presence of either NADP or NAD following the method of Rusvai and Naray-Fejes-Toth [19]. The assay mixture contained sodium phosphate buffer 0.1 M (pH 7.5), 10 nmol/l of [1,2,6,7-^{3}H]corticosterone and 0.25 mmol/l of either NADP or NAD. After 10 min at 37°C, cell lysate was added to the solution. Preliminary studies indicated the amount of protein to be used for each experimental condition to maintain the rate of reaction in the linear range of the product formed and to not exceed 50% of the substrate conversion. After 2 h of incubation, the reaction was arrested and the steroids were extracted by adding 2 volumes of ice-cold ethyl acetate. The organic layer was separated and evaporated under nitrogen. The steroid residue was dissolved in 30 μl of methanol containing 20 μg each of unlabeled corticosterone and 11-dehydrocorticosterone. The mixture was quantitatively transferred to thin-layer plates and developed in chloroform:methanol (9:1; v/v). Reductase activity was assayed in a similar manner by measuring the conversion of [1,2,6,7-^{3}H]11-dehydrocorticosterone to corticosterone in the presence of NADPH or NADH. Areas corresponding to corticosterone and 11-dehydrocorticosterone were visualized under UV light, cut out, transferred to vials containing liquid scintillant and counted in a β-counter. Kinetic parameters were calculated following the procedure described above using concentrations of [1,2,6,7-^{3}H]corticosterone of 5, 10, 20, 50, 100 and 200 nmol/l, with a saturating concentration of NAD as cofactor (0.25 mmol/l). For inhibition experiments, T-47D cell homogenates were incubated with different concentrations of carbenoxolone (Sigma) and 11-dehydrocorticosterone added from stock solutions in water and ethanol, respectively; 11β-HSD activity was determined as described above.

2.2.3. Steroid treatments

To evaluate the effects of steroids on 11β-HSD activity, T-47D cells were seeded onto six well plates at a concentration of 3×10^5 cells/well and grown to about 80% confluence. Before steroid treatment, cells were washed with sterile PBS and switched to RPMI-1640 phenol red-free with 2% FBS pre-treated with dextran-coated activated charcoal (medium A). After 48 h, confluent cultures received fresh medium A containing the steroids added from 1000× stock solutions in ethanol or ethanol alone as vehicle control and the cultures returned to the incubator. At specified periods of experimental incubation, the medium was removed, the cells harvested and 11β-HSD activity determined as described above.

2.2.4. Protein estimation

Cell protein content was estimated by the Bradford's method [20] using bovine serum albumin as standard. Triton X-100 at a low concentration (0.2%) did not interfere with the assay.

2.2.5. Oligodeoxynucleotides

The following gene-specific primers were used for amplification by polymerase chain reaction (PCR): 11β-HSD1 5′ primer was 5′-TCGGATGGCTTTTATG-3′, the 3′ primer was 5′-ACTTGCTTGCAGAATAGG-3′ and the expected size of the amplified fragment was 571 base pairs (bp). 11β-HSD2 5′ primer was 5′-ACGCAGGCCACAATGAAGTAG-3′, the 3′ primer was 5′-GCAGCCAGGCTGGATGATG-3′ and the expected size of the reaction product was 293 bp [18]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′ primer was 5′-ACGGATTTGGTCGTATTGGGC-3′; the 3′ primer was 5′-AAATTCGTTGTCATACCAGG-3′. The expected size of the reaction product was 929 bp [15].

2.2.6. Determination of relative 11β-HSD mRNA levels by RT-PCR

2.2.6.1. Reverse Transcriptase (RT). Total RNA was isolated from cultured cells following the procedure described by Chomczynski and Sacchi [21] (RNAzol-B, Cinna/Biotech Laboratories, Friendswood, TX, USA). RNA integrity was tested by agarose gel electrophoresis in the presence of formaldehyde 2.2 mol/l. First strand cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase (GeneAmp Kit, Perkin Elmer, Norwalk, CT, USA) as previously described [15]. One μg of total RNA was diluted in Tris–HCl 10 mmol/l, KCl 50 mmol/l, $MgCl_2$ 5 mmol/l (pH 8.3), containing 50 U of M-MLV reverse transcriptase, 20 U of placental RNase inhibitor, deoxy-NTPs (dNTPs; 1 mmol/l each of dGTP, dATP, dTTP and dCTP), oligo d(T) primers 2.5 μmol/l, in 20 μl of volume. The mixture was incubated at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min in a programmable thermal cycler (Perkin Elmer). For each RT, a blank was prepared using all the reagents except RNA, which was substituted by water.

2.2.6.2. PCR. Two μl of RT reaction product were added to a mix containing 5× reaction buffer [Tris–HCl 300 mmol/l, $(NH_4)_2SO_4$ 75 mmol/l, $MgCl_2$ 7.5

mmol/l, pH 8.5], dNTP mixture (final concentration 0.25 mmol/l), 1.0 U cloned *Thermus aquaticus* DNA polymerase (Life Technologies, Milan, Italy), and either 11β-HSD1, 11β-HSD2 or GAPDH primers (final concentration 0.4 μmol/l) in a volume of 50 μl and the mixture was overlaid with two drops of mineral oil. PCR incubation was carried out in a programmable thermal cycler (Eppendorf, Netheler, Germany). Amplifications were carried out for 1 min at 94°C, 1 min at 53°C (11β-HSD1) or 63°C (11β-HSD2, GAPDH) and 1 min at 72°C followed by a final 10 min at 72°C [18]. For each incubation, a blank for both sets of primers was prepared using 2 μl of the corresponding RT blank. Semi-quantitative evaluation of 11β-HSD2 mRNA levels in hormone-treated and control cultures was carried out following the method described by Tremblay et al. [22] with minor modifications. The RT products were used as a template for the co-amplification of 11β-HSD2 and the GAPDH cDNA as described above, except that the number of amplification cycles used, determined on the basis of preliminary experiments, was within the range of the exponential phase of reaction. For each amplification, one-fifth of the PCR solution was added to 3 μl of gel loading solution [sucrose 40% (p/v), bromophenol blue 0.05% (p/v), SDS 0.5% (p/v) and EDTA 0.1 mol/l] and fractionated by electrophoresis in a 1.8% agarose gel. Gels were stained with ethidium bromide 0.5 μg/ml, destained and photographed.

2.2.6.3. Statistics. Two-way ANOVA was used to compare: (a) values of samples incubated without cofactor and those of samples incubated in the presence of cofactor and (b) values of cultures treated with the various steroids and values of control cultures. $p < 0.05$ was considered significant.

3. Results

3.1. 11β-HSD activity of T-47D cells

The characteristics of 11β-HSD activity of T-47D are described in Fig. 1. The cofactor preference of the enzyme was determined measuring the conversion of corticosterone to 11-dehydrocorticosterone in the absence of added cofactor or in the presence of 0.25 mmol/1 of NAD or NADP. As shown in panel A, no significant difference in the conversion rate was observed when the cell homogenate was incubated in the absence of cofactor (1.0 ± 0.2 pmole/h mg protein; mean ± SE) or in the presence of NADP (0.98 ± 0.3 pmole/h mg protein). Conversely, exogenous NAD increased 11-dehydrocorticosterone formation up to 3.1 ± 0.44 pmole/h mg protein ($p < 0.05$). To evaluate the reductase activity of 11β-HSD of T-47D, cell homogenates were incubated with 11-dehydrocorticosterone and NADH or NADPH, and in the absence of added cofactor. As shown in panel B, no increase of reductase activity was detectable with either NADH or NADPH.

The kinetics of corticosterone oxidation to 11-dehydrocorticosterone by 11β-HSD was measured from the initial velocities of the conversion at substrate concen-

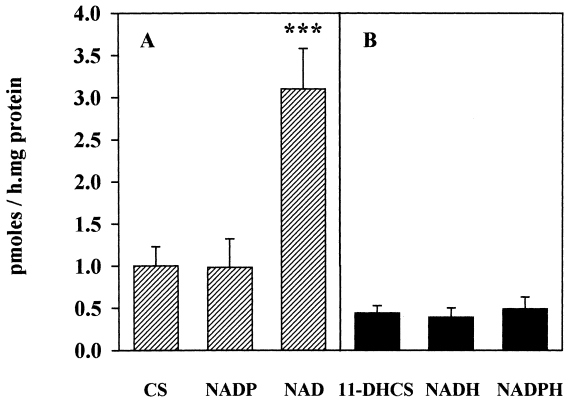

Fig. 1. (A) Cofactor dependence of 11β-HSD. T-47D cell homogenates were incubated in the absence of added cofactor or in the presence of 0.25 mmol/l of NAD or NADP with 10 nmol/l [^{3}H]corticosterone (CS) as substrate. (B) Reductase activity of 11β-HSD. Cell homogenates were incubated in the absence of added cofactor or with 0.25 mmol/l of NADH or NADPH and 10 nmol/l [^{3}H]11-dehydrocorticosterone (11-DHCS). Values are mean ± SE; $n = 5$ (CS, NADP); $n = 12$ (NAD); $n = 3$ (11-DHCS, NADH, NADPH); *** $p < 0.05$ compared to the values of control samples incubated without cofactor.

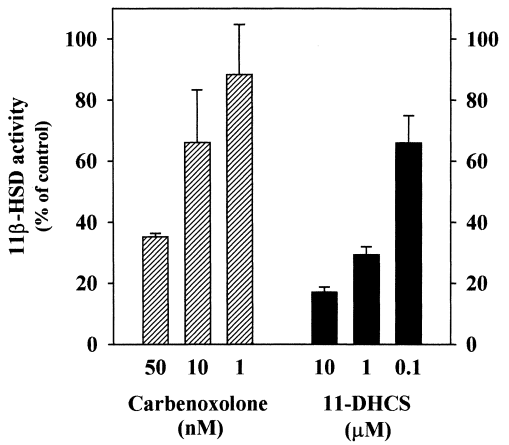

Fig. 2. Inhibition of 11β-HSD activitity of T-47D cells by carbenoxolone and 11-dehydrocorticosterone. Cell homogenates were incubated with 10 nmol/l [^{3}H]corticosterone and 0.25 mmol/l of NAD in the presence of carbenoxolone or 11-dehydrocorticosterone (11-DHCS) at the concentrations indicated. Values are expressed as percent inhibition when compared with control samples incubated without inhibitors. Values are mean ± SD from quadruplicate cultures.

trations ranging from 5 to 200 nmol/l, with a saturating concentration of NAD as cofactor (0.25 mmol/l). The K_m value for corticosterone determined from Lineweaver-Burk plots of the data was 79.8 ± 3.0 nM (n = 3).

Fig. 2 shows the results of the incubation of T-47D cell homogenates in the presence of two inhibitors of 11β-HSD2 activity, the glycyrrhetinic acid synthetic derivative, carbenoxolone, and the end product 11-dehydrocorticosterone. In cell homogenates incubated with increasing concentrations of the inhibitor, a dose-dependent decrease of 11β-HSD activity was observed, with the highest inhibition by carbenoxolone at the concentration of 50 nmol/l (35% of control value) and by 11-dehydrocorticosterone at the concentration of 10 μmol/l (17% of control value).

3.2. 11β-HSD mRNA analysis

To evaluate the steady-state levels of 11β-HSD mRNA in T-47D cells, total RNA was analyzed by RT-PCR. As shown in Fig. 3, in the presence of 11β-HSD2 primers, amplification yielded a single band of 293 bp corresponding to the 11β-HSD2 product (lane 3). In contrast, no product was obtained using 11β-HSD1 primers (lane 2). These results demonstrate a correspondence between 11β-HSD catalytic activity and mRNA levels in T-47D cells.

3.3. Effects of steroids on 11β-HSD activity

The effect of steroid hormones on 11β-HSD activity of T-47D cells is shown in Fig. 4. In cells exposed for 24 h to 10 nmol/l of estrone (E_1), 10 nmol/l of E_2 or 100 nmol/l of MPA, neither E_1 nor E_2 affected 11β-HSD activity, whereas MPA caused a 11-fold increase compared with that of control incubation ($p < 0.001$).

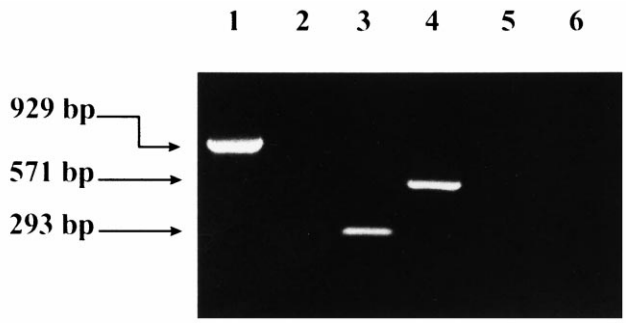

Fig. 3. RT-PCR analysis of 11β-HSD mRNA levels in T-47D cells. One μg of total RNA was reverse transcribed and amplified in the presence of a control gene (GAPDH), 11β-HSD1 or 11β-HSD2 primers. Thirty-five cycles of amplification were carried out. Lane 1 = Control gene (GAPDH); lane 2 = 11β-HSD1; lane 3 = 11β-HSD2; lane 4 = 11β-HSD1 positive control (cDNA); lanes 5 and 6 = blank. The size of the products is indicated.

When E_2 (10 nmol/l) was added to the medium together with MPA (100 nmol/l) the stimulatory effect was not significantly higher than with the progestin alone (data not shown). In cells exposed for 24 h to MPA plus RU486 (1 μmol/l), the antiprogestin counteracted any MPA-induced increase of 11β-HSD activity by reducing the activity to basal levels, while RU486 alone did not exert any agonistic effect on the enzyme activity. In contrast to the marked effect of MPA, the synthetic glucocorticoid dexamethasone (DEX) used at equimolar concentration (100 nmol/l) did not alter 11β-HSD activity. Exposure of the cells to different concentrations of E_2 (1–100 nmol/l) for an extended period (up to 6 days) failed to increase 11β-HSD activity (data not shown).

3.4. Dose and time dependence of the response

Table 1 (left panel) presents levels of 11β-HSD in T-47D cells cultured for 24 h with different concentrations of MPA (1–100 nmol/l). The progestin exerted a clear dose-response effect to enhance 11β-HSD activity compared with levels measured in incubation receiving vehicle alone; an increase of activity was observed at the lowest concentrations tested (1 nmol/l).

To measure the time-course expression of 11β-HSD activity in T-47D cells, daily activity levels of 11β-HSD were measured in cells maintained in control medium or treated with 100 nmol/l of MPA. As shown in Table 1 (right panel), MPA elevated 11β-HSD activity by 24 h, followed by an increase over the subsequent 72 h of incubation.

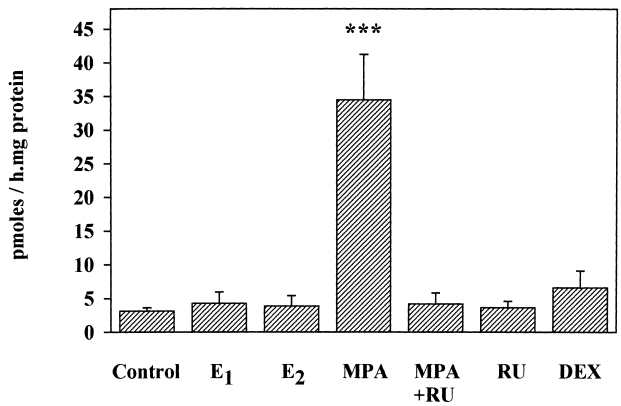

Fig. 4. Steroid-regulated T-47D cell 11β-HSD expression. Cultured T-47D cells were incubated in medium A containing vehicle control, E_1 (10 nmol/l), E_2 (10 nmol/l), MPA (100 nmol/l), MPA plus RU486 (RU; 1 μmol/l), RU486 (RU; 1 μmol/l) or DEX (100 nmol/l) for 24 h. Levels of 11β-HSD were measured in cell homogenates. Values are mean ± SE; n = 12 (control); n = 3 (E_1, E_2, MPA + RU;RU); n = 8 (MPA); n = 4 (DEX); *** $p < 0.001$ compared to control values.

Table 1
Dose and time response of 11β-HSD activity to MPA in T-47D cells[a]

Dose (nmol/l)			Time (h)			
1	10	100	24	48	72	96
2.8 ± 0.6	8.9 ± 1.4	15.1 ± 1.3	7.8 ± 2.7	12.2 ± 0.1	14.2 ± 1.9	23.6 ± 0.4

[a] Cultured cells were incubated in medium A containing vehicle control or MPA. For dose-response experiments, cells received the steroid at the indicated concentrations for 24 h (left panel). For time-course experiments, cells were maintained in the presence of the steroid at the concentration of 100 nmol/l for 24, 48, 72 and 96 h (right panel). Levels of 11-HSD2 were measured in cell homogenates as described in Section 2. The results, mean ± SD from triplicate cultures, normalised to cell protein, are expressed as fold of control value.

3.5. Effect of steroids on 11β-HSD mRNA levels

In order to evaluate whether steroid-mediated modification of 11β-HSD activity corresponded to changes in steady-state levels of 11β-HSD mRNA, RNA extracted from cells treated with either control vehicle, MPA or MPA plus RU486 was analyzed by semi-quantitative RT-PCR. As shown in Fig. 5 (upper panel), treatment of T-47D cells with MPA resulted in an increase of 11β-HSD2 mRNA (lane 2). In cells treated with MPA plus RU486, the antiprogestin blocked the MPA-dependent enhancement of 11β-HSD2 mRNA levels (lane 3).

4. Discussion

The aim of the present study was to investigate the effects of ovarian steroids on 11β-HSD activity in human breast cancer cells. To reproduce the mechanism of the hormonal control of 11β-HSD in vitro, we developed a tissue culture system using the well-differentiated breast cancer cell line T-47D. These cells have been previously used to study the effects of steroids on the expression of several breast epithelial cell proteins, including steroid metabolizing enzymes [8,23,24], and are well characterized for both their response to hormones and receptor content. In particular, T-47D cells contain estrogen and progesterone receptors [25,26] and are responsive to both steroids [27,28]. Moreover, their progesterone receptor levels are constitutively high and independent of estrogen controls [29]. Thus, T-47D cells represent a useful model to examine the effects of estrogens and progestins on specific breast epithelial cell endpoints.

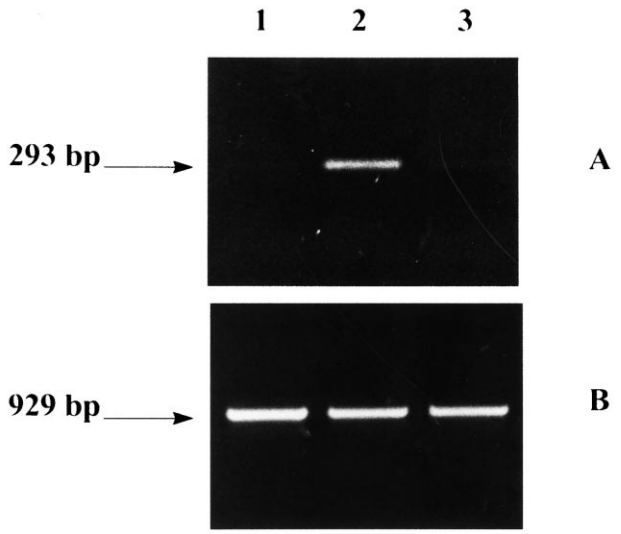

Fig. 5. Steroid effects on levels of 11-HSD2 mRNA as demonstrated by RT-PCR. One microgram of total RNA from cultured T-47D cells incubated with vehicle (lane 1), MPA (100 nmol/l; lane 2), or MPA + RU486 (1 μmol/l; lane 3) for 24 h in medium A was reverse transcribed and amplified in the presence of 11β-HSD2 primers (upper panel) or GAPDH primers (lower panel). Twenty-five cycles of amplification were carried out. The size of the products is indicated.

The first part of our study focused on the evaluation of the characteristics of 11β-HSD of T-47D cells. Our results indicate that 11β-HSD2 is the 11β-HSD isoform expressed by the cells in steady-state conditions. Thus, 11β-HSD of T-47D cells preferred NAD rather than NADP as cofactor and showed a predominant dehydrogenase activity. In addition, the K_m values for corticosterone were similar to those reported for the cloned human 11β-HSD2. Finally, enzyme activity was inhibited by both carbenoxolone and the end product 11-dehydrocorticosterone [10,30]. RT-PCR analysis of 11β-HSD mRNA levels supported these results. When total RNA from T-47D cells was examined, only a band corresponding to the 11β-HSD2 product was obtained, while no 11β-HSD1 mRNA was detected.

Previous investigations have examined the expression of 11β-HSD in normal and cancer human breast epithelial cells. NAD-dependent 11β-HSD activity has been described, although not characterized, in human breast cancer cell lines where the enzyme is thought to play a role in modulating the effects of glucocorticoids on cell growth [13]. Moreover, 11β-HSD2 immunoreactivity has been demonstrated in normal and malignant human breast epithelial cells [12]. The results of the present study supplement these observations, giving evidence of a predominant, if not exclu-

sive, 11β-HSD2 expression in human breast cancer cells.

We then determined the effects of progestins and estrogens on 11β-HSD2 expression of T-47D cells. In cultures exposed to MPA for 24 h, 11β-HSD2 activity increased up to a mean of 11-fold. Induction of enzyme activity was time- and dose-dependent, with response at concentrations spanning the physiological range of circulating progesterone, from the luteal phase to pregnancy. Moreover, as demonstrated by RT-PCR analysis, MPA was a potent inducer of 11β-HSD2 gene expression, as it increased the steady-state levels of 11β-HSD2 mRNA. T-47D cells are known to metabolize progesterone very rapidly [31]. Thus, in our study, this hormone was substituted with the synthetic progestin MPA. However, since MPA also exerts glucocorticoid action in breast cancer cells [32], we tested the effects of a synthetic glucocorticoid, DEX, on T-47D cells. In contrast to the marked increase of the expression levels in response to MPA, 11β-HSD2 was refractory to DEX. In addition, RU486, a potent antagonist of the progesterone receptor, counteracted the progestin-mediated effects, strongly suggesting that MPA induction of 11β-HSD2 activity is exerted specifically via progesterone receptor. Considered together, these results demonstrate that MPA acts as a pure progestin in inducing 11β-HSD2 activity in T-47D cells.

The MPA-dependent induction of 11β-HSD2 activity shown in the present work is in agreement with recent studies that demonstrated progestin enhancement of 11β-HSD2 expression in normal and cancer endometrial cells [15,33]. However, our results differ from the data reported by Sun et al. [34] in human placenta syncytiotrophoblasts in vitro. These authors observed a significant reduction in levels of 11β-HSD2 mRNA after treatment with progesterone, an effect reversed by the receptor antagonist RU486. In light of these findings, discussions on the mechanisms underlying regulation of 11β-HSD2 by steroid hormones should take into consideration tissue-specific differences in the control of 11β-HSD2 expression by progestins. As reported in the results section, in contrast with the profound effect of progestins, neither E_1 nor E_2 directly affected 11β-HSD2 activity in T-47D cells. This is in line with the lack of correlation between estrogen receptor and 11β-HSD2 expression reported in breast cancer tissues [12]. Furthermore, the observation that treatment with E_2 did not augment the progestin-dependent induction of 11β-HSD2 is consistent with the lack of estrogen controls of progesterone receptor in T-47D cells.

The results of the present report clearly indicate that progestin stimulates 11β-HSD2 activity in the T-47D cell line. Extrapolation of these findings to the in vivo status suggests that plasma progesterone levels or tissue uptake can directly affect 11β-HSD2 activity in human breast cancer cells. It must be noted that our results, as those obtained with any other in vitro model, may not completely reflect the behavior of the cell in vivo. However, several factors suggest the relevance of the progestational regulation of 11β-HSD2 activity in breast tissues. Firstly, 11β-HSD2 is expressed in T-47D as well as in breast epithelial cells in vivo [12]. Secondly, 11β-HSD2 activity of T-47D cells does not differ in its characteristics from that of the cloned human enzyme [10,30]. Finally, the concentrations of progestin required to exert effects on 11β-HSD2 activity are in the physiological range of circulating progesterone.

The progestational induction of 11β-HSD2 activity demonstrated in the present report raises the question as to its biological significance in view of the functions of the enzyme in the human breast epithelial cell. Glucocorticoids can affect cancer cell growth by both a direct action on cell proliferation and indirect effects due to changes in the hormonal environment, and such mechanisms have been extensively investigated in vitro using human breast cancer cell lines. Thus, the direct growth inhibitory effect of glucocorticoids has been demonstrated on ZR-75-1 and MCF-7 [5,6] and more recently, in Con-8 human breast cancer cells [7]. Moreover, the response of aromatase activity to glucocorticoids in MCF-7 and T-47D cell lines has suggested the important role of these hormones in the regulation of estrogen synthesis by breast carcinoma cells [8]. Within this context, the recent observation of Hundetmark et al. is noteworthy, as these authors showed that inhibition of 11β-HSD enhances the antiproliferative effect of glucocorticoids on MCF-7 and ZR-75-1 cell lines, thus, indicating that modulation of enzyme activity can significantly influence the hormonal response of human breast cancer cells [13]. The involvement of progestins in the biological action of glucocorticoids on breast carcinoma cells is not known. The effects of these hormones on cell proliferation are contradictory since they have been reported to cause stimulation, inhibition, or no changes in growth rate [35]. It is recognized, however, that the response of breast cancer cells to progestins comprises changes in the expression levels of steroid-metabolizing enzymes, such as 17β-hydroxysteroid dehydrogenase, steroid sulfatase and sulfotransferase [35]. The results reported here indicate the existence of a previously undocumented mechanism of action of progestins on breast cancer cells based on the modulation of the intracellular levels of biologically active glucocorticoids exerted via induction of 11β-HSD2 activity.

The enzyme 11β-HSD2 is abundantly expressed in classical mineralocorticoid target tissues, such as the kidney and colon, where it confers mineralocorticoid specificity by converting physiological glucocorticoids

to their inactive metabolites and allowing aldosterone to bind to the non-selective mineralocorticoid receptor (MR) [36]. Previous investigations have demonstrated the presence of MR in the ductal epithelium of excretory and secretory organs where aldosterone is thought to promote electrolyte transport across the epithelial surface [37]. In human breast cancer, the presence of MR has been previously described by Martin et al. [38]. Recently, Sasano et al. have demonstrated colocalization of MR and 11β-HSD2 in normal and malignant human breast tissues. These authors proposed that in breast epithelial cells, 11β-HSD2 could play an important role to ensure selective binding of aldosterone to its physiological receptor [12]. There is a limited number of data in literature concerning the biological effects of mineralocorticoids in the breast, and it is still not clear whether MR has a functional role in epithelial cells. Martin et al. [38] hypothesized, however, that in the mammary gland, aldosterone could act by affecting ionic movements through the gland epithelium. Re-examination of these previous results in the light of the present data suggests the possibility that alterations in electrolyte homeostasis can be associated with the response of breast epithelial cells to progestins.

5. Conclusion

This report presents data on the hormonal regulation of 11β-HSD activity of human breast cancer cells using the T-47D cell line as an in vitro model. We have demonstrated that these cells express the 11β-HSD2 isoform and that the enzyme is induced by progestin. Further investigations are required to confirm the functioning of this mechanism in all progesterone-responsive breast epithelial cells.

Acknowledgements

The authors thank Dr. Agarwal of the University of Texas for the kind supply of 11β-HSD1 cDNA. RU486 was provided by Research Biochemicals International as part of the Chemical Synthesis Program of the National Institute of Mental Health, Contract N01MH30003. This work was supported by a grant from the Ferrero Foundation (to M.C.).

References

[1] M. Tsai, J.H. Clark, W.T. Schrader, B.W. O'Malley, Mechanisms of action of hormones that act as transcription regulatory factors, in: J.D. Wilson, D.W. Foster, H.M. Kronenberg, P. Reed Larsen (Eds.), Williams Textbook of Endocrinology, 9th ed, WB Saunders Company, Philadelphia, 1998, p. 55.

[2] K.M. Darcy, S.F. Shoemaker, P.P. Lee, B.A. Ganis, M.M. Ip, Hydrocortisone and progesterone regulation of the proliferation, morphogenesis, and functional differentiation of normal rat mammary epithelial cells in three dimensional primary culture, J. Cell. Physiol. 163 (1995) 365–379.

[3] W.R. Lyons, C.H. Li, R.D. Cole, R.E. Johnson, The hormonal control of mammary growth and lactation, Recent. Prog. Horm. Res. 14 (1958) 219–250.

[4] R. Denamur, Reviews of the progress of dairy science. Section A. Physiology. Hormonal control of lactogenesis, J. Dairy Res. 38 (1971) 237–264.

[5] M. Lippman, G. Bolan, K. Huff, The effects of glucocorticoids and progesterone on hormone-responsive human breast cancer in long-term tissue culture, Cancer Res. 36 (1976) 4602–4609.

[6] C.K. Osborne, M.E. Monaco, C.R. Kahn, K. Huff, D. Bronzert, M.E. Lippman, Direct inhibition of growth and antagonism of insulin action by glucocorticoids in human breast cancer cells in culture, Cancer Res. 39 (1979) 2422–2428.

[7] L. Goya, A.C. Maiyar, Y. Ge, G.L. Firestone, Glucocorticoids induce a G1/G0 cell cycle arrest of Con8 rat mammary tumor cells that is synchronously reversed by steroid withdrawal or addition of transforming growth factor-alpha, Mol. Endocrinol. 7 (1993) 1121–1132.

[8] C.M. Ryde, J.E. Nicholls, M. Dowsett, Steroid and growth factor modulation of aromatase activity in MCF7 and T47D breast carcinoma cell lines, Cancer Res. 52 (1992) 1411–1415.

[9] G.M. Tannin, A.K. Agarwal, C. Monder, M.I. New, P.C. White, The human gene for 11β-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization, J. Biol. Chem. 266 (1991) 16653–16658.

[10] A.L. Albiston, V.R. Obeyesekere, R.E. Smith, Z.S. Krozowski, Cloning and tissue distribution of the human 11β-hydroxysteroid dehydrogenase type 2 enzyme, Mol. Cell. Endocrinol. 105 (1994) R11–17.

[11] S.J. Quirk, J.A. Slattery, J.W. Funder, Epithelial and adipose cells isolated from mammary glands of pregnant and lactating rats differ in 11β-hydroxysteroid dehydrogenase activity, J. Steroid Biochem. Mol. Biol. 37 (1990) 529–534.

[12] H. Sasano, A.R. Frost, R. Saitoh, G. Matsunaga, H. Nagura, Z.S. Krozowski, S.G. Silverberg, Localization of mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase type II in human breast and its disorders, Anticancer Res. 17 (1997) 2001–2007.

[13] S. Hundertmark, H. Buhler, M. Rudolf, H.K. Weitzel, V. Ragosch, Inhibition of 11β-hydroxysteroid dehydrogenase activity enhances the antiproliferative effect of glucocorticosteroids on MCF-7 and ZR-75-1 breast cancer cells, J. Endocrinol. 155 (1997) 171–180.

[14] K. Yang, M.W. Khalil, B.J. Strutt, D.W. Killinger, 11β-Hydroxysteroid dehydrogenase 1 activity and gene expression in human adipose stromal cells: effect on aromatase activity, J. Steroid Biochem. Mol. Biol. 60 (1997) 247–253.

[15] F. Arcuri, C. Monder, C.J. Lockwood, F. Schatz, Expression of 11β-hydroxysteroid dehydrogenase during decidualization of human endometrial stromal cells, Endocrinology 137 (1996) 595–600.

[16] R.E. Smith, L.A. Salamonsen, P.A. Komesaroff, K.X. Li, K.M. Myles, M. Lawrence, Z. Krozowski, 11β-Hydroxysteroid dehydrogenase type II in the human endometrium: localization and activity during the menstrual cycle, J. Clin. Endocrinol. Metab. 82 (1997) 4252–4257.

[17] P.J. Burton, Z.S. Krozowski, B.J. Waddell, Immunolocalization of 11β-hydroxysteroid dehydrogenase types 1 and 2 in rat uterus: variation across the estrous cycle

and regulation by estrogen and progesterone, Endocrinology 139 (1998) 376–382.
[18] F. Arcuri, S. Sestini, L. Paulesu, L. Bracci, A. Carducci, F. Manzoni, C. Cardone, M. Cintorino, 11β-Hydroxysteroid dehydrogenase expression in first trimester human trophoblasts, Mol. Cell. Endocrinol. 141 (1998) 13–20.
[19] E. Rusvai, A. Naray-Fejes-Toth, A new isoform of 11β-hydroxysteroid dehydrogenase in aldosterone target cells, J. Biol. Chem. 268 (1993) 10717–10720.
[20] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[21] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[22] J. Tremblay, D.B. Hardy, L.E. Pereira, K. Yang, Retinoic acid stimulates the expression of 11β-hydroxysteroid dehydrogenase type 2 in human choriocarcinoma JEG-3 cells, Biol. Reprod. 60 (1999) 541–545.
[23] M. Poutanen, V. Isomaa, K. Kainulainen, R. Vihko, Progestin induction of 17β-hydroxysteroid dehydrogenase enzyme protein in the T-47D human breast-cancer cell line, Int. J. Cancer 46 (1990) 897–901.
[24] M.L. Hsieh, M.C. Charlesworth, M. Goodmanson, S. Zhang, T. Seay, G.G. Klee, D.J. Tindall, C.Y. Young, Expression of human prostate-specific glandular kallikrein protein (hK2) in the breast cancer cell line T47-D, Cancer Res. 57 (1990) 2651–2656.
[25] D.K. Biswas, L. Averboukh, S. Sheng, K. Martin, D.S. Ewaniuk, T.F. Jawde, F. Wang, A.B. Pardee, Classification of breast cancer cells on the basis of a functional assay for estrogen receptor, Mol. Med. 4 (1998) 454–467.
[26] J.F. Savouret, F. Fridlanski, M. Atger, M. Misrahi, R. Berger, E. Milgrom, Origin of the high constitutive level of progesterone receptor in T47-D breast cancer cells, Mol. Cell. Endocrinol. 75 (1998) 157–162.
[27] S. Classen, K. Possinger, R. Pelka-Fleischer, W. Wilmanns, Effect of onapristone and medroxyprogesterone acetate on the proliferation and hormone receptor concentration of human breast cancer cells, J. Steroid Biochem. Mol. Biol. 45 (1998) 315–319.
[28] M. Gupta, A. McDougal, S. Safe, Estrogenic and antiestrogenic activities of 16α- and 2-hydroxy metabolites of 17beta-estradiol in MCF-7 and T47D human breast cancer cells, J. Steroid Biochem. Mol. Biol. 67 (1998) 413–419.
[29] M.B. Mockus, B.A. Lessey, M.A. Bower, K.B. Horwitz, Estrogen-insensitive progesterone receptors in a human breast cancer cell line: characterization of receptors and of a ligand exchange assay, Endocrinology 110 (1982) 1564–1571.
[30] R.W. Brown, K.E. Chapman, Y. Kotelevtsev, J.L. Yau, R.S. Lindsay, L. Brett, C. Leckie, P. Murad, V. Lyons, J.J. Mullins, C.R. Edwards, J.R. Seckl, Cloning and production of antisera to human placental 11β-hydroxysteroid dehydrogenase type 2, Biochem. J. 313 (1996) 1007–1017.
[31] K.B. Horwitz, M.B. Mockus, A.W. Pike, P.V. Fennessey, R.L. Sheridan, Progesterone receptor replenishment in T47D human breast cancer cells. Roles of protein synthesis and hormone metabolism, J. Biol. Chem. 258 (1983) 7603–7610.
[32] T.M. Ewing, L.J. Murphy, M.L. Ng, G.Y. Pang, C.S. Lee, C.K. Watts, R.L. Sutherland, Regulation of epidermal growth factor receptor by progestins and glucocorticoids in human breast cancer cell lines, Int. J. Cancer 44 (1989) 744–752.
[33] A. Darnel, T.K. Archer, K. Yang, Estrogen and progesterone stimulate the expression of 11-hydroxysteroid dehydrogenase type 2 in the Ishikawa human endometrial cell line. Proceedings of the 81st Annual Meeting of The Endocrine Society, 1999 (Abstract P3-271).
[34] K. Sun, K. Yang, J.R.G. Challis, Regulation of 11-hydroxysteroid dehydrogenase type 2 by progesterone, estrogen and the cyclic adenosine 5′-monophosphate pathway in cultured human placental and chorionic trophoblasts, Biol. Reprod. 58 (1998) 1379–1384.
[35] J.R. Pasqualini, J. Paris, R. Sitruk-Ware, G. Chetrite, J. Botella, Progestins and breast cancer, J. Steroid Biochem. Mol. Biol. 65 (1998) 225–235.
[36] P.C. White, T. Mune, A.K. Agarwal, 11β-Hydroxysteroid dehydrogenase and the syndrome of apparent mineralocorticoid excess, Endocr. Rev. 18 (1997) 135–156.
[37] H. Sasano, K. Fukushima, I. Sasaki, S. Matsuno, H. Nagura, Z.S. Krozowski, Immunolocalization of mineralocorticoid receptor in human kidney, pancreas, salivary, mammary and sweat glands: a light and electron microscopic immunohistochemical study, J. Endocrinol. 132 (1992) 305–310.
[38] P.M. Martin, P.H. Rolland, J.P. Raynaud, Macromolecular binding of dexamethasone as evidence for the presence of mineralocorticoid receptor in human breast cancer, Res. 41 (1981) 1222–1226.